Dihydrofolate reductase and membrane translocation:

Evolution of a classic experiment

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As any PhD student or postdoc, who scrambles to write and submit a publication worried about being scooped, can testify science is a fast moving endeavor. Taking into account our limited time and the ever increasing pace with which scientific studies are published few students and postdocs (and PIs as a matter of fact) have time to keep up with the current literature. As a consequence "reading" a manuscript often means just skimming through the abstract, having a glimpse at the figures and in some cases searching the PDF file for keywords of immediate interest. Here I would like to argue that despite these constraints reading the scientific literature should go beyond current papers and also value old classical publications. However, it can be difficult to motivate students and postdocs to read old landmark publications, that report groundbreaking discoveries and opened up new avenues of research. Many think that, while these papers are of historic interest, they are outdated and have little to contribute for scientists expected to use cutting edge methods to produce high impact publications.

However, fact is classic papers still have a lot to offer. For once they withstood the test of the time, which means we know the reported results are correct and reproducible. This is unfortunately not the case for many high impact publications today (1), which due to the hype of selling it to the highest impact factor journal may contained "massaged" data in order tell a cool story which in reality may be much less clear cut than reported. Furthermore, classic scientific publications often impress by a conceptual clarity and in many cases simplicity. They offer insight into how best science should be practiced. These are qualities I miss in many of today's papers published in high impact journals that contain exceedingly large data sets from high through put analyses and a dozen or more supplementary figures that no reader (or reviewer) is able to digest.

In the following I will discuss a 31 year old paper I consider to be such a classic study. It reported a groundbreaking discovery in the field of protein translocation, namely that that posttranslational import of mitochondrial proteins requires unfolding of the substrate (2).

Personal reflections

The reason I chose this specific paper are personal: 31 years ago I was a PhD student working on the cytoskeleton of the parasitic protozoan *Trypanosoma brucei* in the lab of Thomas Seebeck at the University of Bern. My PhD was going well, I knew I wanted to stay in academia and started to think about postdoc positions. This was when I read the paper "Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria" by Eilers and Schatz (2) for the first time. I was so fascinated by the paper, that I knew protein translocation across membranes would be the scientific field I wanted to work on in my future career. Shortly afterwards I applied for a postdoc position in the Schatz lab at the Biocenter in Basel. This was quite naive as I was not aware at that time how famous Jeff Schatz was. Very surprisingly in retrospect, since I wasn't too well prepared for the interview, I was offered a position and joined the Schatz lab in 1987 to start working on mitochondrial protein import in yeast. To my disappointment the famous Eilers and Schatz experiment was not of great relevance for my postdoc project. Little did I know that 31 years later a variation of the experiment would help me to solve an important scientific question in my own lab.

In the following I will discuss the classic Eilers and Schatz paper. In order to illustrate its long lasting impact I will present four of the many publications in which variations of the experiment were used to answer important biological questions. I would like to add the disclaimer that the choice of these papers might not be representative as it was also guided by my personal interests.

To unfold, or not to unfold, that is the question

In vitro import systems allowing energy-dependent import of added substrate proteins into isolated mitochondria were already well established in 1986 (3, 4). However, not much was known about the mechanism of the process. At that time the general rule was that proteins are co-translationally translocated across membranes, although there was

a growing number of examples, including essentially all proteins imported into mitochondria, were protein folding occured prior to membrane translocation (5-7). Thus, a key question was whether mitochondrial protein import requires unfolding of the transported substrate. How could this be addressed experimentally? The basic concept behind the study of Eilers and Schatz is as beautifully simple as the consequences were far-reaching (Fig. 1A). The authors had the ingenious insight that high affinity binding of a ligand to the active center of an enzyme may stabilize its structure and prevent it from being unfolded. The enzyme they used in the study was cytosolic dihydrofolate reductase (DFHR) of mouse, the ligand the folate analogue methotrexate (MTX); a drug used to treat cancer and as an immunosuppressant. They expressed a chimaeric protein consisting of a N-terminal presequence of the mitochondrial protein cytochrome oxidase subunit 4 (COX4) that was fused to mouse DFHR in *E. coli* and purified it. Addition of this fusion protein (COX4-DHFR) to isolated mitochondria of yeast resulted in efficient import of the chimaeric protein and concomitant processing of the presequence. This was expected, since it had already been shown that a N-terminal presequence is sufficient to target non-mitochondrial proteins into the matrix of mitochondria (8). However, when the same experiment was carried out in the presence of MTX import was abolished. Moreover DHFR became partially protease-resistant, even though it was not imported into mitochondria and thus in principle accessible to the protease (Fig. 1A). Careful control experiments showed that this was due to the fact that in the presence of MTX the enzyme became stabilized in its 3D conformation, creating an import intermediate spanning both mitochondrial membranes. The compelling conclusion of this elegant experiment was that translocation of proteins across the mitochondrial membranes indeed requires unfolding of the cargo proteins. The study therefore indicated that the mitochondrial protein import system must include an unfolding "enzyme", which later was shown to consist of mitochondrial heat shock protein 70 that is peripherally associated with the inside of the inner membrane (9, 10). Moreover, the paper had impact well beyond protein translocation as it was recognized that active unfolding of proteins may also be required for other cellular processes such as proteolytic clearing of cytosolic proteins (11).

Catch me if you can

The Eilers and Schatz experiment provided the foundation for another highly influential study that lead to the first identification of the protein import channel in the

mitochondrial outer membrane in 1989 (12). - Vestweber and Schatz expressed and isolated a modified DHFR containing a single C-terminal cysteine, to which a trifunctional synthetic cross-linker was added using an N-hydroxy succinimide group. The maleimide on the other side of the molecule was crosslinked to bovine pancreatic trypsin inhibitor (BPTI), a small protein containing three disulfide bridges. The cross-linker contained a branch consisting of a photoactivatable group. When the resulting chimaeric protein, COX4-DHFR-cross-linker-BPTI, was added to energized isolated mitochondria, the DHFR and the cross-linker moieties were imported into the organelle (note that there was no MTX present in this experiment) (Fig. 1B). However, due to internal disulfide bridges the BPTI could not be unfolded, analogous to the DHFR bound to MTX. As a consequence an intermediate was formed that was stuck in the import channel. After Illumination a small fraction of the stuck chimaeric precursor protein was converted into a product of higher molecular weight, indicating that the photoactivatable group cross-linked the substrate to the import machinery. Further laborious experiments lead to the identification of the protein to which the import substrate was covalently linked. This protein, termed import site protein of 42 kDa (ISP42), was the first discovered component of the membrane-bound mitochondrial protein import machineries. It was later renamed Tom40 and shown to be a β-barrel protein that forms the pore through which proteins are translocated across the outer membrane. Moreover, its discovery open the way to the characterization of the entire translocase complex of the mitochondrial outer membrane (TOM complex), that consists of seven subunits and that mediates import of essentially all mitochondrial proteins (13, 14).

To unfold, or not to unfold, that is the question - part 2

It had been suggested that peroxisomes, membrane bound organelles involved in oxidative processes, in contrast to mitochondria are able to import fully folded and even multimeric proteins (15). Häusler et al. decided to use DHFR and MTX to test whether this also applies for glycosomes, a peroxisome-like organelle found in *T. brucei* and its relatives (16). Their elegant in vivo study, published in 1996 (17), can be best summarized by the experiment where the localization of a DHFR variant containing both an N-terminal mitochondrial and a C-terminal peroxisomal targeting signal (PTS1), typical for peroxisomal and glycosomal matrix proteins, was analyzed in *T. brucei* (Fig. 1C). As expected a fraction of the fusion protein was imported into mitochondria

whereas another fraction was recovered in the glycosomes. However, addition of aminopterin, a membrane-permeable analogue of MTX, to the cell culture lead to a more than two-fold decrease of the N-terminally processed form of the fusion protein, indicating that less of it was imported into mitochondria. Moreover, sucrose gradient analyses showed that in the presence of aminopterin the mitochondrial fraction of the fusion protein decreased, whereas the fraction associated with glycosomes slightly increased. Thus, aminopterin which stabilizes the 3D conformation of the DHFR greatly reduced mitochondrial import of the fusion protein but did not affect its import into glycosomes. The most parsimonious explanation of these results is that mitochondrial protein import requires unfolding of the transported protein, whereas in the case of the glycosomes fully folded proteins can be imported.

Catch me if you can - part 2

The preliminary culmination of the Eilers and Schatz experiment was reached with the publication by Shiota et al. in 2015, in which the path the presequence takes when it crosses the β-barrel protein Tom40 is resolved at near atomic resolution (18, 19). Whereas Vestweber et al. (12) used a cross-linker attached to the substrate, Shiota et al. (18) probed the yeast mitochondrial import pore by placing an unnatural photoactivatable amino acid into the pore itself. They introduced this crosslinker into 108 different positions (!) of the 387 amino acid long Tom40. Mitochondria were isolated from the resulting cell lines and subjected to in vitro import assays using two types of DHFR fusion proteins as substrates. As expected addition of MTX resulted in the accumulation of substrates in the Tom40 channel. In the two model substrates tested DHFR was either C-terminally fused to a N-terminal presequence or to a hydrophobic mitochondrial carrier protein. On activation by light both substrates were crosslinked to Tom40, but only when the photoactivatable amino acid was facing the interior of the β-barrel pore. Moreover, using a computer generated model of Tom40 it was shown that the positively charged presequence followed a path of aligned negatively charged patches in the interior of the Tom40 pore, whereas the mitochondrial carrier protein was threaded through the pore by a different route interacting mainly with hydrophobic patches.

The study also provided insight into the general architecture of the TOM complex since depending on the position of the photoactivatable amino acid Tom40 was not only crosslinked to the arrested substrates but also to other TOM complex subunits. In the

resulting model three Tom40 molecules are linked to each other by three Tom22 subunits, each of which binds two Tom40 molecules. Moreover, the small Tom subunits Tom5, Tom6 and Tom7 were shown to bind at the periphery of the pore molecules.

In summary the study by Shiota et al. represents an amazing tour de force that provides unprecedented molecular details on the protein import mechanism and on the TOM complex architecture at near atomic resolution.

And now for something completely different

A couple of years ago my lab decided to characterize the mitochondrial protein import system of *T. brucei*. Previous bioinformatic analyses indicated that it must be quite unique (20). This was surprising and highly interesting, however it also complicated the characterization of the system.

That was when we realized that doing our own variant of the Eilers and Schatz experiment, the one that prompted me to study mitochondrial biogenesis in the first place 31 years ago, could help us to identify the unique subunits of the trypanosomal protein import machineries. It was known that expression of DHFR fused to a N-terminal presequence caused the accumulation of an import intermediates at the trypanosomal mitochondrion in the presence of aminopterin (17). Thus, all we had to do was to add an epitope-tag to the C-terminus of the DHFR which allowed us to immunoprecipitate the substrate in the presence and absence of aminopterin from solubilized mitochondria. Subsequently, differential proteomic analysis combined with stable isotope labeling with amino acids in cell culture (SILAC) was used to identify all proteins that were selectively co-isolated with the import-arrested substrate stuck in the import channel (21). The approach was highly successful: it did not only recover all 7 previously characterized subunits of the unique trypanosomal outer membrane protein translocase (14), but it also identified the six integral membrane proteins that build up the trypanosomal inner membrane translocase (TIM complex). Moreover, using a carrier protein that was arrested in the import pathway we furthermore showed that *T. brucei*, in contrast to other eukaryotes, has a single TIM complex only that with minor compositional variations mediates import of presequence-containing as well as of carrier proteins (21). - Thus, we have shown that the Eilers and Schatz experiment in combination with cutting edge proteomic methods can be used to characterize a protein import system without prior knowledge of any of its subunits. All one needs to know is

at least one substrate that is transported by the system and that unfolding is required for transport.

Conclusions

I hope that with this review I could convince at least a few readers that it might be worth to read old landmark papers. The Eilers and Schatz publication together with the many other studies derived from it nicely illustrates that variations of classical experiments may be applicable to novel biological questions. Moreover, methodological advances may offer, in some cases decades later, new ways of how the experiment can be exploited. Thus, I am convinced that we are not at the end yet and that future generations of scientists will find yet other ways of how to use DHFR and its ligand to probe protein translocation and to investigate new problems we do not even think about right now.

Figure legends

Fig. 1. Graphical representation of the key experiments presented in the studies discussed in this review. (A) Mitochondrial import of DHFR fused to a mitochondrial presequence was analyzed in the absence and presence of MTX which prevents its unfolding (2). **(B)** An import intermediate based on a chimaeric import substrate, consisting of a N-terminal mitochondrial presequence fused to DHFR that is C-terminally attached to the highly disulfide-linked BPTI via a trifunctional crosslinker, allows the identification of the import machinery by photocrosslinking (12). **(C)** Import of a DHFR variant containing an N-terminal mitochondrial presequence and a C-terminal peroxisomal targeting signal (PTS1) into mitochondria and glycosomes, respectively, was analyzed in the absence and presence of the MTX-analogue aminopterin (17). **(D)** Using MTX-arrested DHFR fused to a mitochondrial presequence in mitochondria containing variants of Tom40 with strategically placed photocrosslinkable amino acids allows to retrace the path of the presequence in Tom40 at near atomic resolution (18). **(E)** Using an aminopterin-arrested C-terminally hemagglutinine epitope (HA)-tagged DHFR fused to a mitochondrial presequence followed by a spacer consisting of the mature part of an imported protein the subunits

of the protein import machineries were identified by immunoprecipitation and subsequent differential mass spectrometric analysis (21).

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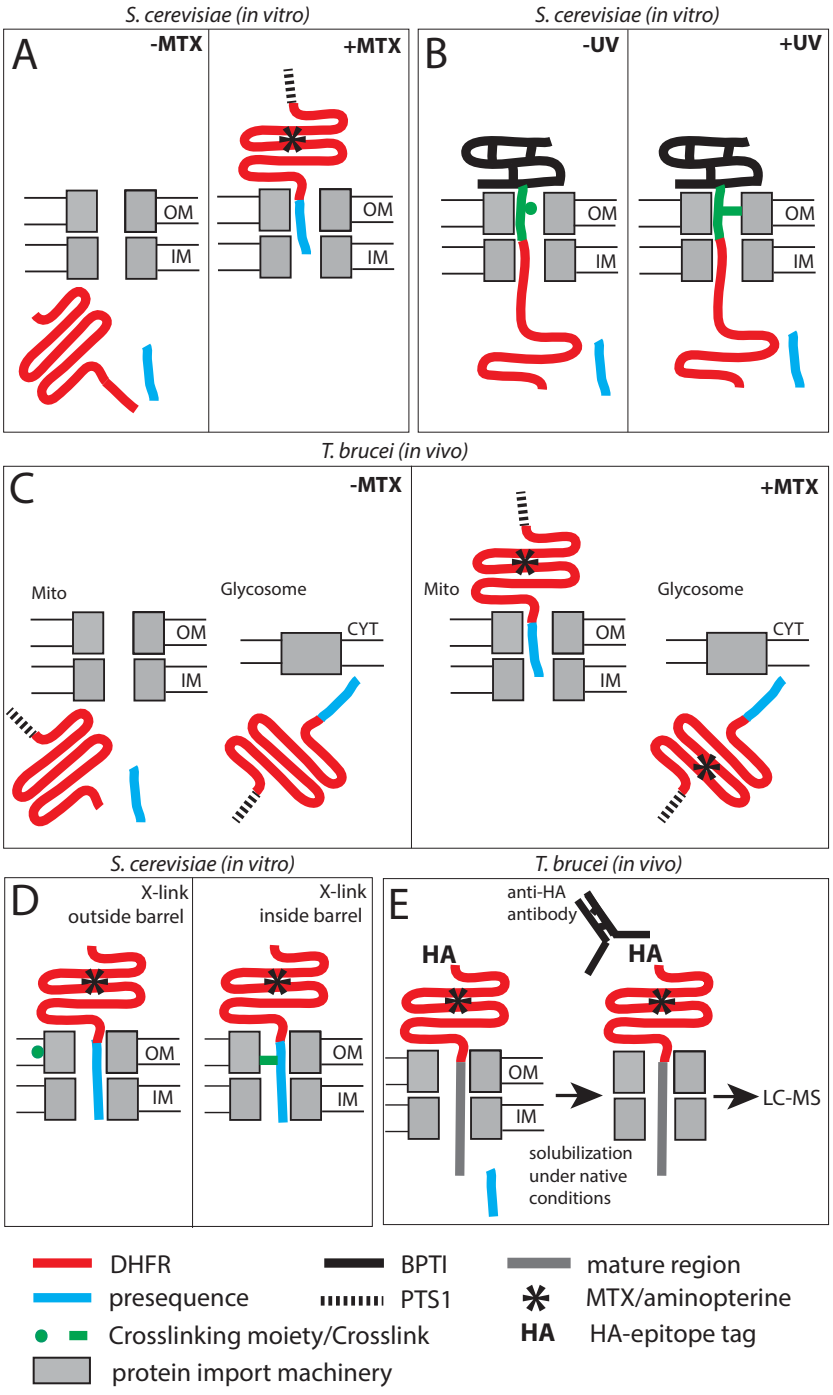
S. cerevisiae (in vitro)
A
-MTX
+MTX
OM
IM
S. cerevisiae (in vitro)
B
-UV
+UV
OM
IM
T. brucei (in vivo)
C
-MTX
+MTX
Mito
Glycosome
OM
IM
CYT
S. cerevisiae (in vitro)
D
X-link outside barrel
X-link inside barrel
OM
IM
T. brucei (in vivo)
E
anti-HA antibody
HA
HA
OM
IM
solubilization under native conditions
LC-MS
DHFR
presequence
Crosslinking moiety/Crosslink
protein import machinery
BPTI
PTS1
mature region
MTX/aminopterine
HA
HA-epitope tag

Figure 1